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The importance of acidic microenvironment in urothelial carcinomas of the bladder: relationship with carbonic anhydrase IX expression and prognostic factors

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ABSTRACT

Aims: Bladder urothelial carcinoma (BUC) is a prevalent malignancy worldwide, ranking 13th in terms of mortality. Several prognostic factors affecting survival have been identified, including histologic grade, invasion of the muscularis propria, tumor diameter, and lymphovascular invasion. However, new markers that will be helpful in diagnosis, treatment and prognosis are still needed. Carbonic anhydrase IX (CA IX) is a tumor-associated cell surface glycoprotein that aids in adaptation to acidosis induced by hypoxia and plays a role in cancer progression.There are few studies on the prognostic impact of CA IX on BUCs. This study aimed to investigate whether CA IX is a promising diagnostic and prognostic biomarker in BUC.

Methods: A retrospective analysis was conducted on 117 cases diagnosed with BUC whithout muscularis propria invasion between September 1995 and January 2023. Transurethral resection (TUR) specimens were examined histological grade, lymphovascular invasion, tumor diameter by a single pathologist. CAIX was performed by immunohistochemical (IHC) method.

Results: Among the 117 patients included in the study, 61 had low-grade tumors, while the remaining 56 had high-grade tumors. CA IX expression exhibited a significant positive correlation with histological grade ($p<0.01$), lymphovascular invasion ($p<0.01$). and tumor diameter ($p<0.01$). Low CA IX staining was observed in three normal tissues and it was found to be a biomarker in the distinction between malignant and benign cases.

Conclusion: CA IX expression is associated with poor prognosis in BUCs as in some other tumors. Evaluation of CA IX staining may be important for patient follow-up and treatment strategies. Adding carbonic anhydrase enzyme inhibitors to chemotherapy regimens could potentially create new treatment options.

Keywords: Bladder, carcinoma, CAIX, prognostic factor

INTRODUCTION

Bladder urothelial carcinoma (BUC) is a disease that is common worldwide and ranks 13th in terms of mortality.[1] According to 2022 GLOBOCAN data, 614.298 people are diagnosed with bladder cancer worldwide each year.[2] Each year, 220.596 people lose their lives to bladder cancer. The majority of patients with BUC are men, and the incidence in men is three times higher than in women. The average age at diagnosis is 73 years. Imaging examinations and cystoscopy are the basic clinical diagnostic methods, and the primary treatment is surgery. Early diagnosis significantly increases treatment results and recovery rates.[3] Therefore, it is important to identify tumor markers for BUC.[4,5] Several prognostic factors affecting survival have been identified, including histologic grade, invasion of the muscularis propria, tumor diameter, and lymphovascular invasion.[6-10] However, new markers that will be helpful in diagnosis and treatment are still needed.

Hypoxia poses a life-threatening condition for all aerobic organisms, and they develop various adaptation mechanisms to survive in such conditions.[11-13] The rapid proliferation of cancer cells increases the oxygen demand, but the vessels supplying oxygen-carrying blood cannot keep up. Consequently, hypoxia occurs in rapidly growing tumor tissues, and tumor cells develop adaptive responses to cope with this stress.[14] Hypoxia can be moderate or severe, acute or chronic, and intermittent or persistent, leading to various cellular responses that promote aggressive tumor phenotypes.[5] At the molecular level, these changes are primarily determined through the remodeling of hypoxia-inducible factor (HIF)-mediated transcriptional

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profiles. HIF targets genes that encode angiogenesis mediators such as vascular endothelial growth factor (VEGF) and VEGF receptors, as well as enzymes involved in the glycolytic pathway such as hexokinase 2, lactate dehydrogenase, and glucose transporters (GLUT-1 and GLUT-3). Additionally, it affects erythropoiesis, vascular remodeling, cell proliferation and viability, cell adhesion, cell-matrix metabolism, and pH regulation.[15,16]

Due to the development of hypoxia and increased energy demand, glycolysis is enhanced, leading to the production of excess acidic metabolic end products such as lactic acid, protons, and carbon dioxide. This activation triggers pH regulatory mechanisms. Intracellular acidosis is usually eliminated by CO_2 diffusion, removal of lactate and protons from the cell, and intake of bicarbonate ions. However, because tumor vessels cannot effectively remove acidic metabolic waste, pericellular acidosis often persists in the tumor microenvironment.[17]

Carbonic anhydrase IX (CA IX) is a tumor-associated cell surface glycoprotein that aids in adaptation to acidosis induced by hypoxia and plays a role in cancer progression. The active site of the CA IX enzyme in the catalytic domain is positioned towards the extracellular space, contributing to pH regulation across the plasma membrane by facilitating CO_2 hydration. This, in turn, enhances CO_2 diffusion and proton mobility in the tumor tissue. Simultaneously, CA IX exacerbates extracellular acidosis, which can activate proteases to degrade the extracellular matrix, promote epithelial-mesenchymal transition and invasion, reprogram metabolism, affect cell adhesion, and stimulate inflammation and angiogenesis. CA IX is more abundant in tumor tissues than in normal tissues.[18,19]

Numerous studies have found that CA IX is a new type of tumor antigen involved in tumor formation and invasion, enabling tumors to maintain high viability under hypoxic conditions. Hypoxia and low-pH environments promote the invasion and metastasis of cancer cells. Several studies have shown that CA IX can be highly expressed in various malignant tumors.[20-22]

There is an increasing focus in the literature on the role of CA IX in cancer development and its prognostic impact. Urothelial carcinomas are one of the common malignancies. Identifying prognostic markers for this aggressive type of cancer is vital to improving patient outcomes and tailoring treatment strategies. In this study, we investigated whether CA IX, an enzyme involved in the regulation of pH balance in cells, was a promising prognostic biomarker in BUC and its prognostic effects.

METHODS

This retrospective study was initiated after obtaining approval from the Ethics Committee of Fırat University (Date: 09.03.2023, Decision No: 2023/04-34). All procedures performed in studies involving human participants were conducted in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or ethical standards. The study focused on patients who were diagnosed as having BUC and were referred to the pathology laboratory of our hospital between September 2018 and January 2023. All samples were obtained through transurethral resection (TUR). The tissues of some cases were not available in the pathology archive. In some cases, there was not enough tissue left for re-evaluation. Therefore, these cases were excluded from the study. Cases with smooth muscle invasion were also excluded from the study.

A single pathologist examined hematoxylin-eosin sections of patient samples and archival materials using a Leica DM 2000 light microscope. The pathologist confirmed tumor type, histologic grade, muscularis propria invasion, and lymphovascular invasion. 117 patients without muscularis propria invasion were included in the study. The diagnosis was made according to World Health organization (WHO) 2016 histopathologic type criteria.[6] Additionally, tumors were classified as low-grade or high-grade according to WHO histologic grading criteria.[7]

Fifty benign bladder biopsies taken for various non-tumor reasons were also determined as a control group to determine the diagnostic effectiveness of CA IX. It was desired to investigate whether CA IX was expressed in benign tissues. Three-micron-thick sections were taken from the paraffin blocks of both the tumor tissues and the control group and placed on the slides. An immunohistochemical (IHC) method was applied to determine CA IX expression. After deparaffinization and treatment with 3% H_2O_2 for 5 minutes, tissue samples were blocked with 10% serum in a blocking solution for 1 hour. They were then incubated overnight at 4°C with anti-CA IX antibodies at a 1/400 dilution in antibody diluent (Leica Prodact code: NCL-L-CAIX-U Clon:TH22).

CA IX exhibits cytoplasmic membrane staining because it is a transmembrane protein. Immunohistochemically positive cells were graded as follows: negative if <10%, low stain if 10-50%, intermediate stain if 51-75%, and high stain if 76-100%.[8]

Demographic, laboratory, and clinical variables of the patients were obtained from the Hospital Information System (HIS) database. Tumor diameter information was collected from radiologic imaging reports and categorized as follows: 0-1 cm, 1.1-2 cm, 2.1-3 cm, and >3 cm.

Statistical Analysis

The data analysis was conducted using the SPSS version 22 software (IBM Corp., Armonk, NY, USA). All analyses were based on the assumption of normality. Descriptive data are expressed as median and mean values for normally distributed variables due to the approximate values of the calculated mode. The Chi-square test and regression analyze were used. A p-value of <0.01 was considered statistically significant.

RESULTS

Of the 117 patients included in the study, 61 had low-grade tumors and the remaining 56 had high-grade tumors. Among the patients, 21 were female and 96 were male, with a mean age of 68 years. Among the women, nine were classified as having low-grade tumors, and 12 had high-grade tumors. Fifty-two men had low-grade tumors and 44 had high-grade tumors.

Negative staining for CA IX was seen in 53 of 61 (88%) low-grade cases. Six cases (10%) showed low staining and two cases (2%) showed intermediate staining. In high-grade cases, 13 (23.2%) showed negative staining, 17 (30.3%) showed low staining, 12 (21.4%) showed intermediate staining, and 14 (25.1%) showed high staining. Regression analysis was performed. Based on the results of the regression analysis conducted to predict the effect of CA IX expression on histologic grade, it was observed that CA IX expression levels have a statistically significant positive

impact on histologic grade. The R-squared value, representing the explanatory power of the model, was calculated as 0.406 (R=0.637, R^2=0.406, p=0.001). This indicates that the intensity of CA IX expression accounts for 40.6% of the variance in histologic grade. In other words, it was observed that as the level of CA IX expression increases, the histologic grade of the tumor also increases (**Table 1**, **Figure**).

Lymphovascular invasion was observed in 11 patients, all of which were classified as high histologic grade. There was no lymphovascular invasion in 89 patients. No lymphovascular invasion was observed in any low-grade cases. Negative CA IX staining was observed in two of the lymphovascular invasion-positive cases, intermediate staining was observed in two cases, and high staining was observed in seven cases. Regression analysis was performed. It was observed that CA IX expression levels have a statistically significant positive impact on lymphovascular invasion. The R-squared value, representing the explanatory power of the model, was calculated as 0.479 (R=0.479, R^2=0.229, p=0.001). This indicates that the intensity of CA IX expression accounts for 22,9% of the variance in lymphovascular invasion. This suggests that as CA IX expression increases, so does the lymphovascular invasion of the tumor (**Table 1**).

In the examination of 53 cases of CAIX-negative low-grade tumors, the tumor diameter was found to be between 0-1 cm in 7 cases, between 1.1-2 cm in 44 cases, and between 2.1-3 cm in 2 cases. In 6 low-grade urothelial carcinoma cases showing low staining, the tumor diameter was found to be between 1.1-2 cm in 4 cases and between 2.1-3 cm in 2 cases. In both cases showing intermediate staining, the tumor diameter was measured to be between 2.1-3 cm. Among the 13 high-grade urothelial carcinoma cases showing negative staining, one had a tumor diameter of 0-1 cm, eight had diameters of 1.1-2 cm, and four had diameters of 2.1-3 cm. In the 17 cases with low staining, one had a tumor diameter of 0-1 cm, 11 had diameters of 1.1-2 cm, three had diameters of 2.1-3 cm and two had greater than 3 cm. In 12 cases with intermediate staining, the tumor size was 1.1-2 cm in 4 cases, 2.1-3 cm in 4 cases, and greater than 3 cm in 4 cases. In 14 cases with high staining, 6 cases had a tumor size of 2.1-3 cm, and in 8 cases, the tumor size was greater than 3 cm. In the regression analysis, a weak but statistically significant positive effect of CA IX expression on lymphovascular invasion was identified (R=0.335, R^2=0.112, p=0.01) (**Table 2**).

Table 1. Relationship between CA IX expression and histologic grade, lymphovascular invasion

	Low grade UC	High grade UC	p
Female Male	9 52	12 44	
CA IX staining Negative Grade 1 Grade 2 Grade 3	 53 6 2 -	 13 17 11 14	**p<0.01**
Lymphovascular invasion	-	11 2 cases; CA IX negative 2 cases; CA IX intermediate staining 7 cases; CA IX high staining	**p<0.01**

CA IX: Carbonic anhydrase IX, UC: Urothelial carcinoma

Table 2. Relationship between CA IX expression and tumor diameter

Staining grade	Low grade UC Tumor diameter			High grade UC Tumor diameter			
	0-1 cm	1.1-2 cm	2.1-3 cm	0-1 cm	1.1-2 cm	2.1-3 cm	>3 cm
Negative	7	44	2	1	8	4	0
Grade 1	0	4	2	1	11	3	2
Grade 2	0	0	2	0	4	4	4
Grade 3	0	0	0	0	0	6	8

UC: Urothelial carcinoma

In CA IX staining applied to fifty normal tissues, low staining was observed in three cases. When compared with tumor tissues, a statistically significant difference was observed in the CA IX staining of tumor tissue and normal tissues (p<0.01).

DISCUSSION

CA IX is a tumor-associated cell surface glycoprotein and its expression is primarily induced by the transcription factor HIF-1α during hypoxia. The key features of CA IX are : a) the absence of healthy, nonhypoxic, nontumor tissues (except for some areas of the gastrointestinal tract); b) overexpression in a wide variety of tumors with a hypoxic phenotype;

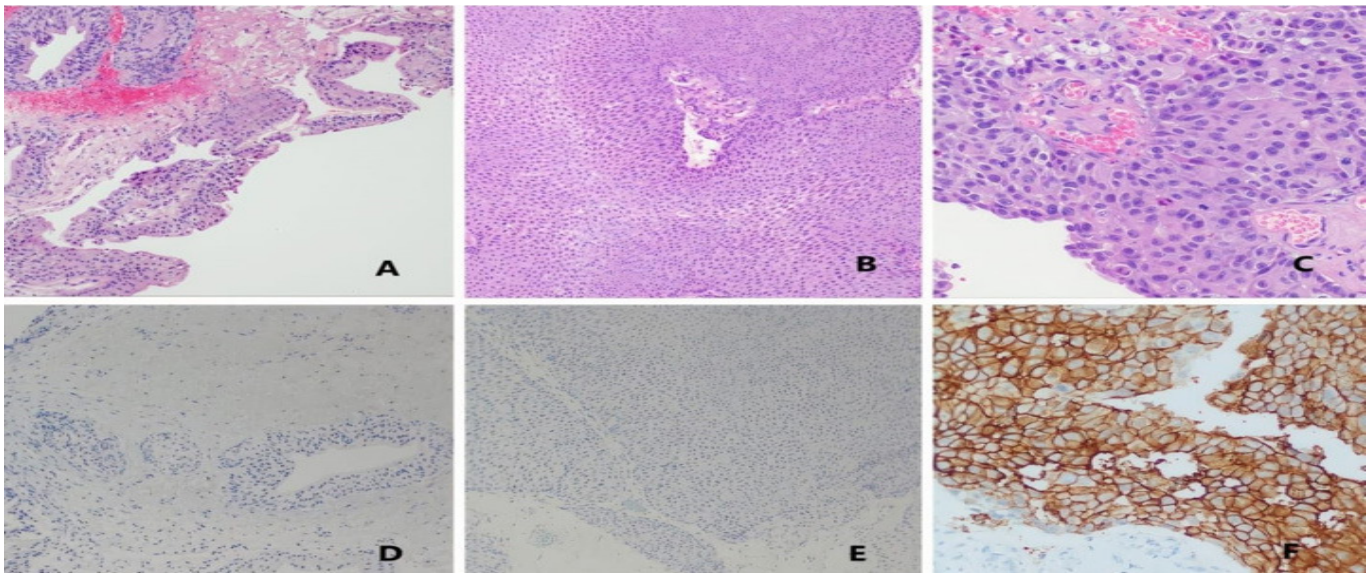

Figure. A. Normal bladder epithelium, **B.** Low grade urothelial carcinoma, **C.** High grade urothelial carcinoma, **D.** Negative staining of CA IX in normal bladder epithelium, **E.** Low grade staining of CA IX in low grade urothelial carcinoma, **F.** High grade staining of CA IX in High grade urothelial carcinoma

c) maintenance of a neutral pH within tumor cells and contribution to the acidosis of the tumor microenvironment; d) it facilitates cell migration and invasion with its ability to regulate pH; e) participation of its proteoglycan (PG)-like domain in tumor cell adhesion and proliferation processes. In summary, CA IX promotes tumor cell survival in hypoxia/acidosis and contributes to the increased ability of tumor cells to migrate, invade, and cure. It metastasizes as reviewed in the literature.[19]

High expression of CA IX is observed in various cancer tissues because it confers resistance to hypoxia and promotes proliferation of cancer cells. Previous studies demonstrated elevated CA IX expression in kidney clear cell carcinoma, prostate cancers, and breast carcinomas. Furthermore, a significant association has been observed between CA IX expression, advanced disease stage, and poor prognosis in certain cancers.[23] A comprehensive meta-analysis of clinical studies results confirmed the significant prognostic importance of IHC diagnostics of CA IX in solid tumors.[24] Patients with high CA IX expression have a higher risk of local failure, disease progression, and a higher risk of metastases developing, independently of tumor type or site. It has been found that the presence of CA IX in tumor tissue may also serve as a predictive marker for radiotherapy and chemotherapy resistance.[25]

In this study, CA IX expression was evaluated in bladder carcinomas without muscularis propria invasion. In our studies including 117 patients, a positive correlation was found between CA IX expression and histologic grade, lymphovascular invasion, and tumor size. Consistent with the literature, as the degree of CA IX expression increased, so did histologic grade and lymphovascular invasion. In the study by Xiang et al.[8] no significant relationship was found between tumor diameter and CA IX expression. These factors have prognostic importance for BUC. When CA IX expression increases, poor prognostic factors increase. CA IX expression has been identified as a diagnostic marker for malignancy.[8] In our study, a statistically significant difference was observed in the CA IX staining of tumor tissue and normal tissues. We think that these data can be used as a diagnostic marker in cases where it is difficult to distinguish malignant from benign.

When studies on CA IX expression in BUCs began to be conducted, Hoskin et al.[26] investigated 64 patients and found that the survival rate in CA IX-positive patients was 35%. In the study conducted by Klatte et al.[27] in 2009, the authors said that the study by Hoskin et al. was the most comprehensive study on this subject. In Klatte et al.'s study, it was shown that there was more CA IX expression in invasive carcinomas than in non-invasive tumors. Additionally, it was observed that CA IX expression increased as histologic grade increased. Also, it was observed that there was no staining in the normal bladder epithelium, as in our study.[27]

In the study conducted by Xiang et al.[8] CA IX expression was detected in 68.1% of 194 patients with urothelial carcinoma. Among the 76 patients who experienced recurrence during their 5-year follow-up, 59 expressed CA IX. In our study, we were unable to access recurrence outcomes because the hospital's information system data did not extend beyond 5 years. In their study on 180 patients, Todenhöfer et al.[28] found that there was more staining in tumor tissue than in normal tissue and that CA IX expression was only associated with molecular subtypes, not with other prognostic parameters. In addition, it was determined that its use in the diagnosis of malignant/benign tumors was limited because staining was also observed in normal tissues in their studies.

In addition to the diagnostic and prognostic importance of CA IX, studies investigating its importance in treatment attract attention. According to the current clinical research results, the therapeutic strategy of CAIX has two directions. The first is to inhibit the enzyme activity at the active site of CAIX, including sulfonamides and their thioesters. The second is to use specific monoclonal antibodies to selectively kill tumors with CAIX expression, such as G250/girentuximab and M75.[19,29] In the study conducted by Chen et al.[30] on rats with breast carcinoma, CA IX was found to be quite high in cancer tissues. It was found that tumor diameter decreased and invasion decreased when CA IX inhibitors were used and that the treatment increased apoptosis.[30] There are contradictory reports regarding the response to treatment in BUCs. Some studies reported a positive response to treatment, but other studies observed that bladder tumors were insensitive to the inhibition of CA IX.[31,32]

Limitations

It is worth noting that our study has some limitations, such as the small sample size and the lack of long-term patient follow-up. It is important to conduct further research with larger study groups in order to apply these findings to routine clinical practice.

CONCLUSION

We conclude that CA IX expression is associated with poor prognosis in urothelial carcinomas of the bladder. Evaluating the degree of CA IX staining in TUR-M materials and biopsies is important in terms of prognosis prediction, patient follow-up, and treatment. Therefore, we predict that the addition of carbonic anhydrase enzyme inhibitors to chemotherapy regimens will affect the prognosis.

ETHICAL DECLARATIONS

Ethics Committee Approval

The study was conducted with the permission of Fırat University Non-interventional Researches Ethics Committee (Date: 09.03.2023, Decision No: 2023/04-34).

Informed Consent

Because the study was designed retrospectively, no written informed consent form was obtained from patients.

Referee Evaluation Process

Externally peer-reviewed.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Financial Disclosure

The authors declared that this study has received no financial support.

Author Contributions

All of the authors declare that they have all participated in the design, execution, and analysis of the paper, and that they have approved the final version.

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